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Attorney Docket No.: 10356.000-US

PATENT

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

Mail Stop Provisional Patent Application
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Sir:

This is a request for filing a provisional application under 37 CFR 1.53(c).

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TITLE OF THE INVENTION
Phospholipase Variants

The following application parts are enclosed:

[x] specification 13 pages [] Sequence Listing __ pages

[x] Abstract 1 page [x] Drawings 3 pages

An application data sheet is enclosed.

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PATENT TRADEMARK OFFICE

25908

Please charge the required fee, estimated to be $160, to Novozymes North America, Inc., Deposit Account No. 50-1701. A duplicate of this sheet is enclosed.

Respectfully submitted,

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

EXPRESS MAIL CERTIFICATE

Mail Stop Provisional Patent Application
Commissioner for Patents
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Alexandria, VA 22313-1450

Re: U.S. Provisional Application for
"Phospholipase Variants"
Applicants: Patkar et al.

Sir:

Express Mail Label No. EV 138339476 US

Date of Deposit June 19, 2003

I hereby certify that the following attached paper(s) or fee

1. Filing Under 37 C.F.R. §1.53(c) (in duplicate)
2. Provisional Application
3. Application Data Sheet

are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" under 37 C.F.R. 1.10 on the date indicated above and is addressed to the address indicated above.

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Application Data Sheet

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Title: Phospholipase variants

Field of invention

The present invention relates to a variant enzyme derived from a parent enzyme having phospholipase activity, to a method of increasing the ratio of phospholipase/lipase activity in a variant lipolytic enzyme compared to a parent lipolytic enzyme having phospholipase activity. Also the present invention relates to a method for producing cheese.

Background of the invention

Lipolytic enzymes (such as lipases and phospholipases) are capable of hydrolyzing carboxylic ester bonds in a substrate to release carboxylic acids. The hydrolytic activity on different ester bonds is important for the usefulness of the lipolytic enzyme in various industrial applications.

WO 00/32758 discloses lipolytic enzyme variants having phospholipase activity. WO 98/26057 discloses a *Fusarium oxysporum* phospholipase. WO 01/83770 describes variants. WO 00/54601 describes a process for producing cheese from enzyme-treated cheese milk.

Summary of the invention

The present invention relates to improved enzyme variants having phospholipase activity and which enzymes variants have an increased ratio of phospholipase/lipase activity compared to a parent lipolytic enzyme having phospholipase activity.

In a first aspect the invention relates to a lipolytic enzyme derived from a parent enzyme, wherein the parent enzyme is at least 50 % identical to SEQ ID No. 1 and the parent enzyme has phospholipase activity, and wherein the variant enzyme has a modified amino acid sequence, said modification comprises a substitution of an amino acid residue corresponding to R84W in SEQ ID No. 1.

In a second aspect the invention relates to a method of increasing the ratio of phospholipase/lipase activity in a variant lipolytic enzyme compared to a parent lipolytic enzyme having phospholipase activity, comprising introducing an amino acid substitution corresponding to R84W in SEQ ID No. 1 in the parent enzyme.

In a third aspect the invention relates to a method for producing cheese, which method comprises the steps of:

a) treating cheese milk or a fraction of the cheese milk with a variant enzyme according to any of the claims 1-3; and

b) producing cheese from the cheese milk, wherein step a) is conducted before and/or during step b).

Brief description of drawings

Figure 1 shows an alignment of fungal lipolytic enzyme sequences: 1. *Absidia reflexa*, 2. *Absidia corymbefera*, 3. *Rhizomucor miehei*, 4. *Rhizopus delemar* (*oryzae*), 5. *Aspergillus niger*, 6. *Aspergillus tubingensis*, 7. *Fusarium oxysporum*, 8. *Fusarium heterosporum*, 9. *Aspergillus oryzae*, 10. *Penicilium camembertii*, 11. *Aspergillus foetidus*, 12. *Aspergillus niger*, 13. *Aspergillus oryzae*, 14. *Thermomyces lanuginosus* (*Humicola lanuginosa*).

Definitions

Identity: The term "at least 50% identical" in the context of the present invention relates to homologous polynucleotides or polypeptides. The degree of identity is at least 50%, more preferably at least 55%, more preferably at least 60%, more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, even more preferably at least 85%, still more preferably at least 90%, more preferably at least 95%, and most preferably at least 98%. Whether two polynucleotide or polypeptide sequences have a sufficiently high degree of identity can suitably be investigated by aligning the two sequences using a computer program known in the art, such as "GAP" provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711)(Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453). Using GAP with the following settings for DNA sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3.

An enzyme which exhibits phospholipase activity may be an enzyme which in the "monolayer phospholipase assay", as described in WO 0032758, has a phospholipase activity of at least 0.25 nmol/min, enzyme dose: 60 μg, at 25°C; more preferably at least 0.40 nmol/min, enzyme dose: 60 μg, at 25°C; more preferably at least 0.75 nmol/min, enzyme dose: 60 μg, at 25°C; more preferably at least 1.0 nmol/min, enzyme dose: 60 μg, at 25°C; more preferably at least

1.25 nmol/min, enzyme dose: 60 µg, at 25°C; and even more preferably at least 1.5 nmol/min, enzyme dose: 60 µg, at 25°C.

Phospholipase activity can also be determined by using a plate assay as described in WO 0032758 or by HPLC or by a phospholipid depletion assay as described below.

Variant enzyme: An enzyme derived from a parent enzyme by introducing at least one modification of the amino acid sequence of the parent enzyme.

Detailed description of the invention

Parent lipolytic enzyme

The parent lipolytic enzyme to be used in the present invention is classified in EC 3.1.1 Carboxylic Ester Hydrolases according to Enzyme Nomenclature (available at http://www.chem.qmw.ac.uk/iubmb/enzyme). More specifically the parent enzyme according to the invention is an enzyme having phospholipase activity. Accordingly the parent enzyme according to the invention could be a phospholipase classified in EC 3.1.1.32 or EC 3.1.1.4.

Lipolytic enzymes not having phospholipase activity can be modified and screened in order to select variants having phospholipase activity as disclosed in WO 00/32758.

In the present invention the parent enzyme is derived from SEQ ID No 1 or a lipolytic enzyme which is at least 50 % identical to SEQ ID No 1, and which parent enzyme has phopholipase activity. The parent enzyme may additionally comprise further modifications in case of SEQ ID No 1 or an enzyme which is at least 50% identical to SEQ ID No 1 but which enzyme does not have phospholipase activity. Such further modifications are described below.

WO 00/32758 also discloses a lipolytic enzyme from *Humicola lanuginosa*, the amino acid sequence of which is shown in sequence nr. 14 in figure 1 and in SEQ ID No 1, as well as various variants of SEQ ID No 1 with phospholipase activity and a method of how to obtain such variants. These variants may be the starting point for the phospholipase variants according to the present invention in which an amino acid substitution at the position corresponding to position 84 in SEQ ID No 1 is introduced. In SEQ ID No 1 the position 84 is an arginine. According to the present invention this arginine is substituted by a tryptophan or a

corresponding substitution at a corresponding position in an enzyme at least 50 % identical to SEQ ID No 1.

Surprisingly the variants of the invention comprising the substitution at position 84 or a position corresponding to position 84 in other phospholipases, e.g. in the sequences shown in sequence 1-13 in figure 1, results in phospholipases with improved properties in applications for preparing cheese.

The variant enzyme according to the invention may comprise further modifications than just the substitution at position 84. Such modifications comprises substitutions which will result in a parent enzyme with phospholipase activity as disclosed in WO 00/32758.

In another embodiment the variant enzyme of the invention therefore comprises substitutions corresponding to D57G, V60G/C/K/R/L/S/Q, D62E/F/W/H/V/L/G/A/P/Q, S83T, S85Y, G91E/A/V/R, L93K, D96W/F/G, E99K, R125K, V203T, V228A, L259V/R/S, F262L, G263Q, L264A, I265T, G266D, T267A, L269N.

The starting parent enzyme for producing the variants of the present invention may also be a phospholipase from *Fusarium oxysporum* which is described in WO 98/26057, and the amino acid sequence of which is shown in the alignment in figure 1, sequence number 7 and in SEQ ID No 2. If the starting point for making the variants according to the invention is SEQ ID No 2, it is evident from the alignment shown in figure 1 that the position corresponding to position 84 is position I81 in SEQ ID No 2.

In addition to the modifications described above in order to arrive at the parent enzyme having phospholipase activity, modifications of the variants according to the invention may additionally comprise modifications such as insertions or deletions. Also modifications at the N- or C-terminus, e.g. by adding residues after position 269 are comprised. Such C-terminal additions could e.g. comprise addition of 270A, 271G, 272G, 273F, 274S, 275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS.
N-terminal additions comprise the addition of the amino acid residues SPIR.

The above mentioned additional modifications such as insertions, deletions and C- and N-terminal modifications should not be considered, when calculating the % identity of the parent enzyme with SEQ ID No 1.

The parent enzyme shown in SEQ ID No 2 was C-terminal processed during expression from *A. oryzae*, and the results indicate that positions 270, 271, 272 or 284 in SEQ ID NO 2 is the most likely C-terminal residue in the expressed mature active enzyme.

Phospholipases has been shown to be able to increase the yield of cheese when added during a cheese making process as described in WO 00/54601. Both phospholipase (PLA1) hydrolysing at the Sn1 position and phospholipase (PLA2) that hydrolyse at the Sn2 position works in this application. Often the PLA1 phospholipases also works on triglycerides found in milk. Lipase activity on short chained lipids gives the cheese a different flavour due to the short chained free fatty acids. This can be a desired flavour, but may also been undesired.

Surprisingly we have seen that to high lipase activity on triglycerides can lead to problems in the cheese-making process, possibly due to the generation of to many free fatty acids. Thus it could be desirable to diminish the lipase activity on triglycerides of a PLA1 phospholipase, such as for example, the parent *Thermomyces lanuginosa* lipase of the invention which is a variant of the lipase shown in SEQ ID No 1 having phospholipase activity.

The variants of the invention has the surprising effect of increasing the ratio of phospholipase/lipase activity in a variant lipolytic enzyme compared to a parent lipolytic enzyme having phospholipase activity. By lipase activity is meant lipase activity on triglycerides. Variants according to the invention therefore have a higher phospholipase/lipase activity ratio compared to the parent enzyme according to the invention. These variants according to the invention surprisingly result in an increased yield and at the same time avoids the changes in taste and smell, which may result from the generation of to many free fatty acids, when lipolytic enzymes are used in the production of cheese. Thus the variants according to the invention combines the desirable effect of having improved yields when applying phospholipases in cheese production and at the same time avoids getting a cheese product having changed properties in terms of smell and taste when phospholipases are applied.

It could also be desirable to increase the ratio of phospholipase/lipase activity of the phospholipase from *Fusarium oxysporum* shown in SEQ ID No 2.

Homology and alignment

For purposes of the present invention, the degree of homology may be suitably determined by means of computer programs known in the art, such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-45), using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

In the present invention, corresponding positions in the lipase sequences of *Absidia reflexa, Absidia corymbefera, Rhizomucor miehei, Rhizopus delemar, Thermomyces lanuginosa (former; Humicola lanuginosa), Penicillium camembertii and Fusarium oxysporum, Fusarium heterosporum, Aspergillus tubingensis, Aspergillus oryzae, Aspergillus foetidus, Aspergillus niger* are defined by the alignment shown in Figure 1.

To find the homologous positions in lipase sequences not shown in the alignment, the sequence of interest is aligned to the sequences shown in Figure 1. The new sequence is aligned to the present alignment in Fig. 1 by using the GAP alignment to the most homologous sequence found by the GAP program. GAP is provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-45). The following settings are used for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

Use of variant

The use of phospholipases in the production of cheese products has been described in WO 00/54601. The phospholipases applied in WO 00/54601 result in an increased yield in the cheese production as well as in improving the stability of the fat in the cheese. The variant enzymes according to the present invention are also suitable for the treatment of cheese and can be applied as described in WO 00/54601 in a process for producing cheese. The variants of the invention will result in an increased yield and at the same time avoids the changes in taste and smell, which may result from the generation of to many free fatty acids, when the variant lipolytic enzymes of the invention are used in the production of cheese, e.g. in a process or method as described in WO 00/54601.

In a further embodiment the invention therefore relates to a method for producing cheese, which method comprises the steps of:

a) treating cheese milk or a fraction of the cheese milk with a variant enzyme according to the invention; and

b) producing cheese from the cheese milk, wherein step a) is conducted before and/or during step b).

Examples

Example 1. Construction of variants having a increased phopholipase/lipase activity ratio compared to the parent enzyme.

Variants according to the invention were constructed as described in WO 00/32758. Examples of a variant enzymes derived from SEQ ID No 1 according to the invention are given in Table 1 below.

Table 1.

Variant	Mutation (numbering according to SEQ ID No 1)
1	**R84W**,D96W,E99K,G263Q,L264A,I265T,G266D,T267A,L269N,270A,271G,272G,273F,274S,275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
2	**R84W**,G91E,D96W,E99K,G263Q,L264A,I265T,G266D,T267A,L269N,270A,271G,272G,273F,274S,275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
3	V60G,D62E,**R84W**,G91A,D96F,E99K,G263Q,L264A,I265T,G266D,T267A,L269N
4	**R84W**,G91V, D96F,E99K,G263Q,L264A,I265T,G266D,T267A,L269N
5	**R84W**,G91R,L93K,D96G,E99K,G263Q,L264A,I265T,G266D,T267A,L269N,270A,271G,272G,273F,274S,275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
6	V60G,D62F,**R84W**,G91A,D96W,E99K,G263Q,L264A,I265T,G266D,T267A,L269N,270A,271G,272G,273F,274S,275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
7	**R84W**,S85Y,G91A,D96W,E99K,G263Q,L264A,I265T,G266D,T267A,L269N,270A,

	271G,272G,273F,274S,275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEY VKNNQARS
8	**R84W**,G91A,D96W,E99K,L259V,G263Q,L264A,I265T,G266D,T267A,L269N,270 A,271G,272G,273F,274S,275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDK EYVKNNQARS
9	S83T,**R84W**,G91A,D96W,E99K,G263Q,L264A,I265T,G266D,T267A,L269N,270A, 271G,272G,273F,274S,275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEY VKNNQARS
10	V60G,D62W,**R84W**,G91A,D96F,E99K,G263Q,L264A,I265T,G266D,T267A,L269N
11	**R84W**,G91R,D96F,E99K,G263Q,L264A,I265T,G266D,T267A,L269N,270A,271G, 272G,273F,274S,275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNN QARS
12	V6OC,D62H,**R84W**,G91A,D96F,E99K,G263Q,L264A,I265T,G266D,T267A,L269N
13	V60G,D62V,**R84W**,G91A,D96F,E99K,G263Q,L264A,I265T,G266D,T267A,L269N
14	V60K,D62L,**R84W**,G91A,D96F,E99K,G263Q,L264A,I265T,G266D,T267A,L269N
15	V60R,D62L,**R84W**,G91A,D96F,E99K,G263Q,L264A,I265T,G266D,T267A,L269N
16	V60G,D62G,**R84W**,G91A,D96W,V228A,E99K,G263Q,L264A,I265T,G266D,T267 A,L269N,270A,271G,272G,273F,274S,275WRRYRSAESVDKRATMTDAELEKKL NSYVQMDKEYVKNNQARS
17	V60L,D62A,**R84W**,G91A,D96W,E99K,R125K,G263Q,L264A,I265T,G266D,T267A ,L269N,270A,271G,272G,273F,274S,275WRRYRSAESVDKRATMTDAELEKKLN SYVQMDKEYVKNNQARS
18	D62E,**R84W**,G91A,D96W,E99K,G263Q,L264A,I265T,G266D,T267A,L269N,270A, 271G,272G,273F,274S,275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEY VKNNQARS

19	V60S,D62L,**R84W**,G91A,D96F,E99K,F262L,G263Q,L264A,I265T,G266D,T267A, L269N
20	D57G,V60Q,D62P,**R84W**,G91A,D96F,E99K,G263Q,L264A,I265T,G266D,T267A, L269N
21	**R84W**,G91A,D96W,E99K,L259R,G263Q,L264A,I265T,G266D,T267A,L269N,270 A,271G,272G,273F,274S,275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDK EYVKNNQARS
22	S83T,**R84W**,G91A,D96F,E99K,G263Q,L264A,I265T,G266D,T267A,L269N
23	D62Q,**R84W**,G91A,D96W,E99K,G263Q,L264A,I265T,G266D,T267A,L269N,270A ,271G,272G,273F,274S,275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKE YVKNNQARS
24	**R84W**,G91A,D96W,E99K,L259S,G263Q,L264A,I265T,G266D,T267A,L269N,270 A,271G,272G,273F,274S,275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDK EYVKNNQARS
25	**R84W**,G91A,D96W,E99K,V203T,G263Q,L264A,I265T,G266D,T267A,L269N,270 A,271G,272G,273F,274S,275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDK EYVKNNQARS

The above variants were improved compared to the parent enzyme when measuring the ratio of phospholipase acitivty to lipase activity. This was determined by measuring the depletion of phospholipids in milk-fat (%PL depletion) as described below and by measuring the activity (SLU) of the variants on triglyceride by titration with a pH stat as described in WO 00/32758. From this the %PL depletion/SLU ratio was calculated.

Depletion of phospholipids in cream:

Cream was treated with two different amounts of a phospholipase to determine the depletion of phospholipids by the action of the enzyme.

Substrate Preparation and Enzyme/Substrate Reaction.

Cream was standardized to a fat content of 25% (w/w) using skim milk. Two samples of 1 ml each were incubated with 0.02 mg enzyme protein and 0.1 mg enzyme protein per gram of milk fat, respectively, of a phospholipase (Lecitase®, Novozymes A/S, Bagsværd, Denmark) at 35°C for 1.5 hr without shaking. Reactions were stopped by the addition of organic solvent for lipid extraction.

Lipid Extraction.

Total milk lipids were extracted by mixing each sample with 1 ml of water followed by 9 ml of chloroform/methanol (2:1). Samples were mixed vigorously for 1 min and centrifuged at 3000 rpm for 5 min. Six millilitres of the lower organic phase were removed and dried down under vacuum. Samples were reconstituted in 2 ml of chloroform. Each chloroform extract was applied to an aminopropyl SPE column (Phenomenex, Inc.) under vacuum. The column was washed first with 4 ml of chloroform/isopropanol (2:1) to remove neutral lipids and then with 4 ml of diethylether acidified with glacial acetic acid (2% v/v) to remove free fatty acids. Phospholipids were then eluted with 4 ml of methanol, dried down in a rotary evaporator, and reconstituted in 0.6 ml of mobile phase A for HPLC analysis.

HPLC determination of phosphatidylethanolamine (PE)

HPLC was performed on a Agilent 1100 system containing a quaternary pump, degasser, auto sampler, thermo stated column compartment, evaporative light scattering detector (Polymer Laboratories, Inc.), and a personal computer with Agilent ChemStation software. The stationary phase consisted of a Luna Silica (150 x 4.6 mm, 5 µ, 100 A) analytical column and a Security Guard Cartridge (4.0 x 3.0 mm) consisting of the same packing material. Both analytical and guard columns were from Phenomenex (Torrance, CA USA). The mobile phases consisted of an A mixture containing 80% chloroform, 19.5% methanol, 0.5% ammonium hydroxide and a B mixture of 60% chloroform, 34% methanol, 5.5% water, 0.5% ammonium hydroxide. The following linear gradient was utilized: a starting composition of 80% A/20% B was held for 2 min, proceeding to 100% B from 2 min to 14 min; 100% B was maintained from 14 min to 20 min, returning to 80% A/20% B from 20 min to 23 min. The time required to re-equilibrate the column in a sequence of runs was 7 min. With a flow rate of 1.0 ml/min and a column temperature of 30°C, the pressure increased from approximately 43 to 55 bar. The evaporator temperature of the light scattering detector was 80°C and the nebulizer temperature was 42°C. The nebulization gas was nitrogen used at a flow rate of 1.0 SLM. Highly pure

phosphatidylethanolamine (PE) (Avanti Polar Lipids, Inc.) was used as standard. Stock solution was prepared in chloroform in the concentration range of 2-10 mg/ml. HPLC calibrators were prepared from stock solutions by dilution to the appropriate concentration in mobile phase A.

Table 2. Results of example 1

Enzyme dose (mg enzyme protein/g fat)	**% PE Depletion (versus control without phospholipase treatment)**
0.02	80%
0.10	>90% *

*Peak below lowest limit of quantification

Example 2. Evaluation of cheese yield using selected variants of the invention

Variants according to the invention were evaluated in a method of producing cheese with the addition of a phospholipase. The controls were without phospholipase addition.

The following variants according to the invention was tested:
Variant # 2, 4, 5, 8, 9, 10, 16, 22 and 24.

The method was a bench top cheese yield evaluation test and was performed as described below.

Bench top cheese making.

Standard procedure of bench top cheese processing was conducted as follow:

1. Standardize 0.5 kg cheese milk w/ pasteurized skim milk and cream.

2. Prepare a single starter by adding 0.1 g Rhodia LH100 and 0.3 g Rhodia TA061 starter cultures (for mozzarella) to 50 ml of the skim milk and equilibrate to 35°C w/ gentle, continuous stirring.

3. Equilibrate cheese milk to 35°C and add 0.07 mg enzyme protein per g fat, check initial pH and add 5 ml starter to each cheese milk with gentle agitation .

4. When pH reaches 6.45 – 6.50 add 0.5 ml of 10x diluted Chymax (available from Christian

Hansen); stir vigorously for three minutes then remove stirrers from milk, cover water bath and allow milk to coagulate.

5. Cut curd at the appropriate time (30-45 minutes) w/ ½" knives. To determine cutting time, make a downward cut into the curd with knife or spatula. The curd is ready for cutting when the cut separates upon lifting and sharp edges are maintained on the top surface at the edge of the cut.. Allow the curd to rest for 5 minutes then gently and intermittently stir curd to prevent coalescence of curd particles.

6. Increase temperature to 41°C and hold until curd pH reaches 5.65 – 5.70, then drain and pour curd particles into stainless steel bowls. Float bowls in 41°C water bath to maintain curd temperature. Periodically drain excess whey, leaving only enough to cover curds for maintenance of heat.

7. When curd pH ~ 5.25 - 5.3, drain all whey and flood curd w/ D.I. water at 57°C for 5 min. Stretch the curd by hand for ~ 1min in 59°C water, then place the curd in ice water for 15 min and dry blot. Record weight of curd and refrigerate until further analysis.

Result

All the tested variants according to the invention resulted in improved yield compared to the control, when calculated as moisture adjusted yield.

Claims

1. A lipolytic enzyme derived from a parent enzyme, wherein the parent enzyme is at least 50 % identical to SEQ ID No. 1 and the parent enzyme has phospholipase activity, and wherein the variant enzyme has a modified amino acid sequence, said modification comprises a substitution of an amino acid residue corresponding to R84W in SEQ ID No. 1.

2. The variant according to any of the preceding claims, wherein the parent enzyme is derived from the enzyme shown in SEQ ID No 1 by introducing at least one amino acid substitution of an amino acid residue resulting in phospholipase activity of the parent enzyme, which substitution comprises substitutions corresponding to D57G, V60G/C/K/R/L/S/Q, D62E/F/W/H/V/L/G/A/P/Q, S83T, S85Y, G91E/A/V/R, L93K, D96W/F/G, E99K, R125K, V203T, V228A, L259V/R/S, F262L, G263Q, L264A, I265T, G266D, T267A, L269N.

3. A method of increasing the ratio of phospholipase/lipase activity in a variant lipolytic enzyme compared to a parent lipolytic enzyme having phospholipase activity, comprising introducing an amino acid substitution corresponding to R84W in SEQ ID No. 1 in the parent enzyme.

4. A method for producing cheese, which method comprises the steps of:
a) treating cheese milk or a fraction of the cheese milk with a variant enzyme according to any of the claims 1-3; and
b) producing cheese from the cheese milk, wherein step a) is conducted before and/or during step b).

Abstract

The invention relates to a lipolytic enzyme derived from a parent enzyme, wherein the parent enzyme is at least 50 % identical to SEQ ID No. 1 and the parent enzyme has phospholipase activity, and wherein the variant enzyme has a modified amino acid sequence, said modification comprises a substitution of an amino acid residue corresponding to R84W in SEQ ID No. 1. The variants according to the invention combines the desirable effect of having improved yields when applying phospholipases in cheese production and at the same time avoids getting a cheese product having changed properties in terms of smell and taste when phospholipases are applied.

Figure 1.

Alignment of fungal lipolytic enzyme sequences

```
          1                                                                50
seq1  SSSSTQDYRI ASEAEIKAHT FYTALSANA. .......YCR TVIPG.....
seq2  .SSSTQDYRI ASEAEIKAHT FYTALSANA. .......YCR TVIPG.....
seq3  ..SIDGGIRA ATSQEINELT YYTTLSANS. .......YCR TVIPG.....
seq4  .SASDGGKVV AATTAQIQEF TKYAGIAATA .......YCR SVVPG.....
seq5  ....TAGHAL AASTQ.GISE DLYSRL.VEM ATISQAAYAD LCNIPST...
seq6  ....TAGHAL AASTQ.GISE DLYSRL.VEM ATISQAAYAD LCNIPST...
seq7  .......... GVTTTDFSNF KFYIQHGAAA .......YC. .NSEAAAGSK    31
seq8  .......... TVTTQDLSNF RFYLQHADAA .......YC. .NFNTAVGKP
seq9  .......... DIPTTQLEDF KFWVQYAAAT .......YCP NNYVAKDGEK
seq10 .......... DVSTSELDQF EFWVQYAAAS .......YYE ADYTAQVGDK
seq11 .......... SVSTSTLDEL QLFAQWSAAA .......YCS NNID.SKDSN
seq12 .......... SVSTSTLDEL QLFSQWSAAA .......YCS NNID.SDDSN
seq13 .......... DVSSSLLNNL DLFAQYSAAA .......YCD ENLN.STGTK
seq14 .......... EVSQDLFNQF NLFAQYSAAA .......YCG KNNDAPAGTN    33

          51                                                               100
seq1  GRWSCPHCGV AS..NLQITK TFST..LITD TNVLVAVGEK EKTIYVVFRG
seq2  GQWSCPHCDV AP..NLNITK TFTT..LITD TNVLVAVGEN EKTIYVVFRG
seq3  ATWDCIHCDA TE..DLKIIK TWST..LIYD TNAMVARGDS EKTIYIVFRG
seq4  NKWDCVQCQK WVP.DGKIIT TFTS..LLSD TNGYVLRDKQ KTIYLVFRGT
seq5  .......... .......IIK GEKIYNSQTD INGWILRDDS SKEIITVFRG
seq6  .......... .......IIK GEKIYNSQTD INGWILRYC. .NSEAAAGSK
seq7  ITCSNNGCPT VQGNGATIVT SF..VGSKTG IGGYVATDSA RKEIVVSFRG    79
seq8  VHCSAGNCPD IEKDAAIVVG SV..VGTKTG IGAYVATDNA RKEIVVSVRG
seq9  LNCSVGNCPD VEAAGSTVKL SFS.DDTITD TAGFVAVDNT NKAIVVAFRG
seq10 LSCSKGNCPE VEATGATVSY DFS.DSTITD TAGYIAVDHT NSAVVLAFRG
seq11 LTCTANACPS VEEASTTMLL EFDLTNDFGG TAGFLAADNT NKRLVVAFRG
seq12 VTCTADACPS VEEASTKMLL EFDLTNNFGG TAGFLAADNT NKRLVVAFRG
seq13 LTCSVGNCPL VEAASTQSLD EFNESSSYGN PAGYLAADET NKLLVLSFRG
seq14 ITCTGNACPE VEKADATFLY SFE.DSGVGD VTGFLALDNT NKLIVLSFRG    82

          101                                                              150
seq1  TSSIRNAIAD IVFVPVNYPP V...NGAKVH KGFLDSYNEV QDKLVAEVKA
seq2  TSSIRNAIAD IVFVPVNYPP V...NGAKVH KGFLDSYNEV QDKLVAEVKA
seq3  SSSIRNWIAD LTFVPVSYPP V...SGTKVH KGFLDSYGEV QNELVATVLD
seq4  NSFRSAITDI VFNFSDYKPV ...KGAKVHA GFLSSYEQVV NDYFPVVQEQ
seq5  TGSDTNLQLD TNYTLTPFDT LPQCNGCEVH GGYYIGWVSV QDQVESLVKQ
seq6  ITCSNNGCPT VQGNGATIVT SF..VGSKTG IGGYVATDDS SKEIITVFRG
seq7  SINIRNWLTN LDFG.QEDCS L..VSGCGVH SGFQRAWNEI SSQATAAVAS   126
seq8  SINVRNWITN FNFG.QKTCD L..VAGCGVH TGFLDAWEEV AANVKAAVSA
seq9  SYSIRNWVTD ATFP.QTDPG L..CDGCKAE LGFWTAWKVV RDRIIKTLDE
seq10 SYSVRNWVAD ATFV.HTNPG L..CDGCLAE LGFWSSWKLV RDDIIKELKE
seq11 SSTIENWIAN LDFILEDNDD L..CTGCKVH TGFWKAWESA ADELTSKIKS
seq12 SSTIKNWIAD LDFILQDNDD L..CTGCKVH TGFWKAWEAA ADNLTSKIKS
seq13 SADLANWVAN LNFGLEDASD L..CSGCEVH SGFWKAWSEI ADTITSKVES
seq14 SRSIENWIGN LNFDLKEIND I..CSGCRGH DGFTSSWRSV ADTLRQKVED   130
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Fig. 1 cont.

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          151                                                  200
seq1  QLDRHPGYKI VVTGHSLGGA TAVLSALDLY HHGHA....N IEIYTQGQPR
seq2  QLDRHPGYKI VVTGHSLGGA TAVLSALDLY HHGHD....N IEIYTQGQPR
seq3  QFKQYPSYKV AVTGHSLGGA TALLCALDLY QREEGLSSSN LFLYTQGQPR
seq4  LTAHPTYKVI VTGHSLGGAQ ALLAGMDLYQ REPRLSPKNL SIFTVGGPRV
seq5  QVSQYPDYAL TVTGHSLGAS LAALTAAQL. SATYD....N IRLYTFGEPR
seq6  TGSDTNLQLD TNYTLTPFDT LPQCNSCEVH GGYYIGWISV QDQVESLVQQ
seq7  ARKANPSFNV ISTGHSLGGA VAVLAAANLR VGGT.....P VDIYTYGSPR   171
seq8  AKTANPTFKF VVTGHSLGGA VATIAAAYLR KDGF.....P FDLYTYGSPR
seq9  LKPEHSDYKI VVVGHSLGAA IASLAAADLR TKNY.....D AILYAYAAPR
seq10 VVAQNPNYEL VVVGHSLGAA VATLAATDLR GKGYP....S AKLYAYASPR
seq11 AMSTYSGYTL YFTGHSLGGA LATLGATVLR NDGY.....S VELYTYGCPR
seq12 AMSTYSGYTL YFTGHSLGGA LATLGATVLR NDGY.....S VELYTYGCPR
seq13 ALSDHSDYSL VLTGHSYGAA LAALAATALR NSGH.....S VELYNYGQPR
seq14 AVREHPDYRV VFTGHSLGGA LATVAGADLR GNGY.....D IDVFSYGAPR   175

          201                                                  250
seq1  IGTPAFANYV IGT.......  KIPYQRLVHE RDIVPHLPPG AFGFLHAGEE
seq2  IGTPEFANYV IGT.......  KIPYQRLVNE RDIVPHLPPG AFGFLHAGEE
seq3  VGDPAFANYV VST.......  GIPYRRTVNE RDIVPHLPPA AFGFLHAGEE
seq4  GNPTFAYYVE ST.......G  IPFQRTVHKR DIVPHVPPQS FGFLHPGVES
seq5  SGNQAFASYM NDAFQASSPD  TTQYFRVTHA NDGIPNLPPV EQGYAHGGVE
seq6  QVSQFPDYAL TVTGHSLGAS  LAALTAAQL. SATYD....N IRLYTFGEPR
seq7  VGNAQLSAFV SNQ.......  AGGEYRVTHA DDPVPRLPPL IFGYRHTTPE   214
seq8  VGNDFFANFV TQQ.......  TGAEYRVTHG DDPVPRLPPI VFGYRHTSPE
seq9  VANKPLAEFI TNQ.......  .GNNYRFTHN DDPVPKLPLL TMGYVHISPE
seq10 VGNAALAKYI TAQ.......  .GNNFRFTHT NDPVPKLPLL SMGYVHVSPE
seq11 IGNYALAEHI TSQ......G  SGANFRVTHL NDIVPRVPPM DFGFSQPSPE
seq12 VGNYALAEHI TSQ......G  SGANFPVTHL NDIVPRVPPM DFGFSQPSPE
seq13 LGNEALATYI TDQ......N  KGGNYRVTHT NDIVPKLPPT LLGYHHFSPE
seq14 VGNRAFAEFL TVQ......T  GGTLYRITHT NDIVPRLPPR EFGYSHSSPE   219

          251                                                  300
seq1  FWIMK.....  ....DSSLRV CPNGIETDNC SNSIVPFT.. SVIDHLSYLD
seq2  FWIMK.....  ....DSSLRV CPNGIETDNC SNSIVPFT.. SVIDHLSYLD
seq3  YWITD.....  ..NSPETVQV CTSDLETSDC SNSIVPFT.. SVLDHLSYFG
seq4  WIKS......  ..GTSNVQIC TSEIETKDCS NSIVPFT..S ILDHLSYFDI
seq5  YWSV....DP  YSAQNTFVCT GDEVQCCE.A QGGQGVN...  ..NAHTTYF.
seq6  S.NQAFASYM  NDAFQASSPD TTQYFRVTHA NDGIPNLPPA DEGYAHGVVE
seq7  FWLSGGGGDK  VDYTISDVKV CEGAANLG.C NGGTLGL...  DIAAHLHYF.   259
seq8  YWLNG.GPLD  KDYTVTEIKV CEGIANVM.C NGGTIGL...  DILAHITYF.
seq9  YYITA..PDN  TTVTDNQVTV LDGYVNFK.G NTGTSGGLPD  LLAFHSHVWY
seq10 YWITS..PNN  ATVSTSDIKV IDGDVSFD.G NTGTGLPLLT  DFEAHIWYF.
seq11 YWITS..GNG  ASVTASDIEV IEGINSTA.G NAGEATV...  SVLAHLWYF.
seq12 YWITS..GTG  ASVTASDIEL IEGINSTA.G NAGEATV...  DVLAHLWYF.
seq13 YYISS..ADE  ATVTTTDVTE VTGIDATG.G NDGTDGT...  SIDAHRWYF.
seq14 YWIKS..GTL  VPVTRNDIVK IEGIDATG.G NNQPNIP...  DIPAHLWYF.   262
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Fig. 1 cont.

```
          301                                                        350
 5   seq1  MNTGL.CL.. .......... .......... .......... ..........
     seq2  MNTGL.CL.. .......... .......... .......... ..........
     seq3  INTGL.CT.. .......... .......... .......... ..........
     seq4  NEGS..CL.. .......... .......... .......... ..........
     seq5  GMTSGACTW. .......... .......... .......... ..........
10   seq6  YWSV....DP YSAQNTFVCT GDEVQCCE.A QGGQGVN... ..NAHTTYF.
     seq7  QATDA.CNAG GFSWRRYRSA ESVDKR.... .......... ..........   284
     seq8  QSMAT.CAPI AIPWKR.... .......... .......... ..........
     seq9  FIHADACKGP GLPLR..... .......... .......... ..........
    seq10  VQVDAGKGPG LPFKR..... .......... .......... ..........
15  seq11  FAISE.CLL. .......... .......... .......... ..........
    seq12  FAISE.CLL. .......... .......... .......... ..........
    seq13  IYISE.CS.. .......... .......... .......... ..........
    seq14  GLIGT.CL.. .......... .......... .......... ..........   269

20
          351          366
     seq1  .......... ......
     seq2  .......... ......
     seq3  .......... ......
25   seq4  .......... ......
     seq5  .......... ......
     seq6  GMTSGHCTW. ......
     seq7  .......... ......
     seq8  .......... ......
30   seq9  .......... ......
    seq10  .......... ......
    seq11  .......... ......
    seq12  .......... ......
    seq13  .......... ......
35  seq14  .......... ......
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40